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Combined Multilocus Short-Sequence-Repeat and Mycobacterial Interspersed Repetitive Unit–Variable-Number Tandem-Repeat Typing of *Mycobacterium avium* subsp. *paratuberculosis* Isolates▽‡

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Short-sequence-repeat (SSR) sequencing was applied to 127 *Mycobacterium avium* subsp. *paratuberculosis* isolates typed by mycobacterial interspersed repetitive unit–variable-number tandem repeats (MIRU-VNTR) and IS*900* restriction fragment length polymorphism (RFLP). Combined MIRU-VNTR and SSR typing followed by secondary IS*900* RFLP typing is an improved approach to high-resolution genotyping of this pathogen.

Mycobacterium avium subsp. *paratuberculosis* is the etiological agent of Johne's disease or paratuberculosis, a chronic granulomatous enteritidis in ruminants, known since 1895 (11). Paratuberculosis has become a prevalent infectious disease problem for dairy cattle herds, leading to significant economic losses for producers in most developed countries (19), and is suspected to be associated with Crohn's disease in humans (21). An important requirement of European Union or U.S. paratuberculosis control programs is the development of efficient molecular epidemiological methods to trace paratuberculosis outbreaks and interspecies transmission and to study the potential role of wildlife and/or the environmental reservoir.

IS*900* restriction fragment length polymorphism (RFLP) analysis is the most extensively used method for molecular typing of *M. avium* subsp. *paratuberculosis* (4, 26). However, this technique can require months for culturing of the slow-growing mycobacteria prior to DNA purification. Moreover, IS*900* fingerprinting is difficult to standardize and provides insufficient resolution for discriminating different strains of this genetically homogeneous pathogen.

Multiple-locus variable-number tandem repeat analysis (MLVA) is now established for fast, PCR-based typing of many bacteria. MLVA usually indexes copy number polymorphisms by sizing of PCR fragments amplified from targeted variable-number tandem repeat (VNTR) loci (7, 12, 16). Short-sequence-repeat (SSR) typing is a variation which directly analyzes the sequence of simple homopolymeric tracts of single, di- or trinucleotides (3, 6, 8, 10, 17, 18). Classical MLVA and SSR typing results in numerical genotypes, reflecting the repeat copy numbers in the respective markers, which are especially convenient for online comparisons and phylogenetic analyses (2). These methods are particularly informative for highly clonal bacteria, such as *Mycobacterium leprae*, *Mycobacterium ulcerans*, *Bacillus anthracis*, and *Yersinia pestis* (1, 9, 13, 15, 28). An MLVA system based on genetic elements called mycobacterial interspersed repetitive units (MIRUs) (24, 25) has been internationally standardized as an alternative to IS*6110* RFLP analysis for *Mycobacterium tuberculosis* typing (23).

MLVA based on MIRUs (4, 5, 20, 26) and SSR (3, 6, 8, 10, 17, 18) typing has been proposed for *M. avium* subsp. *paratuberculosis*, following bioinformatic scans of the K10 strain genome (14). These schemes target up to 8 MIRU-VNTR (26) and 11 SSR markers (3), respectively. So far, these methods were only separately evaluated for determining the clonal diversity of *M. avium* subsp. *paratuberculosis*. Here, in order to compare their individual and combined performances, we applied SSR typing to a collection of 127 *M. avium* subsp. *paratuberculosis* isolates from different host and geographic origins, selected from a larger set of 183 such isolates previously typed by MIRU-VNTR and IS*900* RFLP (26). These isolates all originated from different farms and were therefore assumed to be epidemiologically unlinked. However, some unidentified transmission links due to cattle trading cannot be excluded.

SSR typing was performed according to the method of Amonsin et al. (3) using BigDye Terminator v3.1 sequencing kits and a 3730XL DNA analyzer (Applied Biosystems, Courtaboeuf, France). The collection studied was divided into four panels (see Table S1 in the supplemental material). Panel 1 was comprised of 40 isolates, which were selected for having different combinations of INMV (INRA, Nouzilly, MIRU-VNTR) and IS*900* RFLP profiles. In this panel, 19 SSR types

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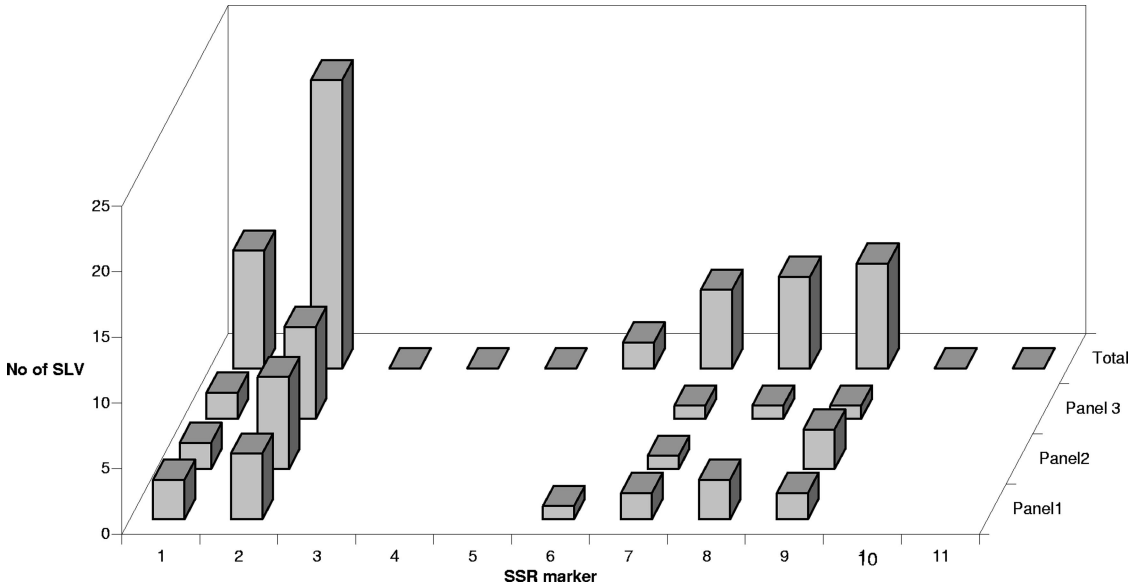

FIG. 1. Distribution of SLVs in SSR loci among *M. avium* subsp. *paratuberculosis* isolates. Events detected among 123 isolates from panels 1 to 3 are shown.

were detected, compared to 13 MIRU-VNTR types and 22 IS*900* RFLP types. When SSR typing and MIRU-VNTR typing were considered in combination, 31 types were obtained. Eight out of the nine MIRU-VNTR cluster types were subdivided by SSR typing, and conversely, the eight SSR cluster types were all subdivided by MIRU-VNTR typing, demonstrating the mutually additive discriminatory powers of both PCR techniques. SSR typing combined with IS*900* RFLP discriminated 38 of the 40 isolates of panel 1, only slightly less than the combination of MIRU-VNTR typing and IS*900* RFLP.

To further evaluate the cumulative discriminatory power of the typing techniques, 43 isolates were selected in panel 2 with the same most-predominant INMV2 profile and the same R01 IS*900* RFLP profile, and 40 isolates were selected in panel 3 with the same second-most-predominant INMV1 profile and the same R01 IS*900* RFLP profile (see Table S1 in the supplemental material) (26). SSR typing subdivided the single combined INMV-RFLP profiles of panels 2 and 3 into 16 types and 14 types, respectively. Because of this selection bias of the strain panels (see below), discriminatory indexes for each method or combination of methods provided in Table S2 in the supplemental material should be considered with caution.

Two methods were used to identify the most informative SSR loci. The relative evolutionary rates of the SSR loci were first analyzed by calculating the frequency of their involvement in discrimination by single-locus variation (SLV), i.e., single-allele differences among closest genotype relatives. SLVs were identified by calculating a minimum spanning tree of SSR-based genotypes, using the software program Bionumerics, version 4.6 (Applied Maths, St-Martens-Latem, Belgium). Since they discriminate closely related strains, loci most frequently involved in SLVs should intrinsically be those with a higher evolutionary rate if the markers are not frequently subject to convergence (independent evolution to the same state). According to this criterion, the top five SSR markers were loci 2, 1, 9, 8, and 7 in decreasing order of SLV frequencies across the three panels (Fig. 1). The allelic diversities (h) of the SSR loci were then calculated as follows: $h = 1 - \Sigma x_i^2\,[n/(n-1)]$, where x_i is the frequency of the ith allele at the locus and n is the number of isolates. From two to six alleles were detected for the six markers that showed variability among the three panels. Consistently, at least three of the above top five loci systematically displayed the highest allelic diversity in any of the three strain panels (see Table S2 in the supplemental material). Interestingly, a substitution of one nucleotide in the repeat unit of marker 8 (G to A) was detected for two isolates. Conversely, SSR loci 3, 4, 5, 10, and 11 did not show any variation among the three strain panels. The variability of MIRU-VNTR markers has already been described for the larger collection by Thibault et al. (26).

The *M. avium* subsp. *paratuberculosis* strain 316F is one of the strains used for vaccination against paratuberculosis worldwide. We applied SSR typing to five isolates of different 316F vaccine batches (see Table S1, panel 4, in the supplemental material), two of which were previously found to be genetically divergent by single-MIRU-VNTR locus and/or one or three IS*900* RFLP band differences (26). These differences were fully corroborated by allelic differences in one or more SSR loci for these two isolates compared to the three others. This observation thus confirms our hypothesis of genetic drift among 316F isolates from different sources, in particular between Weybridge and Mérial batches (26). On the other hand, the consistently complete conservation of the SSR loci, as well as that of the MIRU-VNTR loci and the IS*900* fingerprints, in the three remaining isolates of Mérial origin is consistent with the stability of SSR loci observed after analysis of serial passages or single colonies from individual strains (3, 10). These observations support the potential of the markers involved for epidemiological tracking of longitudinal transmission.

In conclusion, because of their mutually cumulative discriminatory powers, combined MIRU-VNTR and SSR typing is an improved PCR-based approach to high-resolution genotyping

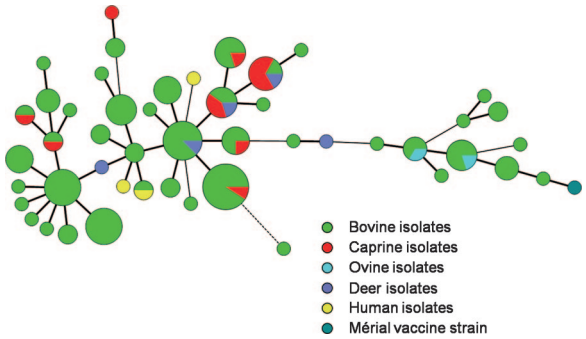

FIG. 2. Minimum spanning tree based on combined MIRU-VNTR and SSR genotypes among *M. avium* subsp. *paratuberculosis* isolates. Circles correspond to the different types identified by the combination of the two methods among 123 isolates from panels 1 to 3, and their sizes are proportional to the number of clustered isolates with an identical combined type. Because of the difficulties of standardizing IS*900* RFLP banding patterns between different RFLP experiments and the distinct nature of the underlying mechanism of polymorphism compared to repeat sequence markers such as MIRU-VNTRs and SSRs, IS*900* RFLP genotypes were not considered here. Thick, thin, and dotted lines between circles correspond to single-, double-, or triple-locus variations between genotypes, respectively.

of *M. avium* subsp. *paratuberculosis*. Our study was not designed to generally compare the resolution powers of the two techniques, since panels 2 and 3 were deliberately biased for including isolates with preidentified identical MIRU-VNTR (and IS*900* RFLP) genotypes. While SSR typing frequently subdivided these prevalent MIRU-VNTR (-IS*900* RFLP)-based clusters, the converse situation may also be expected based on panel 1 analysis results, i.e., MIRU-VNTR typing might frequently subdivide prevalent SSR-based clusters. Regardless, prioritization between MIRU-VNTR and SSR typing may rely on technical and practical considerations rather than on discriminatory power. MIRU-VNTR typing is probably more accessible to most laboratories due to the use of PCR without sequencing, its low cost, and the ease of interpretation. In contrast, even after double-strand sequencing, reading of SSR alleles with >8 or 9 repeats required some expertise and allele assignations were based on a consensus between two readers. Because of too-strong stutter peak effects, alleles with more than 11 repeats in the two most variable SSR loci (1 and 2) could not be reliably read despite repeated sequencing of both DNA strands. Therefore, in order to avoid interpretation errors, we recommend conservatively assigning such alleles as >11, despite some loss of information. Therefore, according to our experience, the most cost-effective and efficient strategy for strain discrimination would consist of using MIRU-VNTR typing first, followed by SSR typing and IS*900* RFLP for potential discrimination of the remaining clustered isolates and optional confirmation of discriminated isolates. Likewise, primary or secondary screens based on SSR typing may use the most-variable loci 1, 2, 7, 8, and 9 in priority. It is noteworthy that loci 1, 2, 8, and 9 were also identified as the most informative SSR markers in a collection of *M. avium* subsp. *paratuberculosis* isolates from the United States (10), which is encouraging for future standardization. Finally, although the majority of our strains were from French and bovine origins, no obvious correlations were found between these and other geographic or host origins and MIRU-VNTR-SSR genotype groups (Fig. 2). Importantly, our collection was comprised only of "C" or cattle phenotype *M. avium* subsp. *paratuberculosis* strains, even those isolated from ovine hosts, and did not include any representative of the rare "S" or sheep phenotype. Consistently, neither multilocus sequence analysis (27) nor large sequence polymorphism analysis (22) revealed any correlation between host provenance and "C"-type genotypes. Taken together, these observations indicate extensive interspecies transmission of *M. avium* subsp. *paratuberculosis*, which in turn underlines the need for improved epidemiological control to prevent further spread from the paratuberculosis reservoir(s).

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